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Chemical properties of O-antigenic lipopolysaccharides isolated from Vibrio parahaemolyticus 02 and 09, in particular, the separation and identification of 2-keto-3-deoxy-5-phosphoryl-octonate found in their strong acid-hydrolysates

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Chemical properties of O-antigenic lipopolysaccharides isolated from Vibrio parahaemolyticus O2 and O9, in particular, the separation and identification of 2-keto-3-deoxy-5-phosphoryl-octonate found in their strong acid-hydrolysates

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A degradation study was carried out on lipopolysaccharides (LPS) isolated from *Vibrio parahaemolyticus* O2 and O9. On Sephadex G-50 gel filtration of "degraded polysaccharide" (DPS) from the S-form strains, no such peak was observed that represents polymeric O-specific side chains with atached core which is generally eluted in void volume. The result suggests that the O-specificities of these *V. parahaemolyticus* are associated with the structure and composition of the core region of their LPS molecules and/or that their O-specific chains are very short.

A Weissbach's periodate-thiobarbituric acid-reaction positive substance (X) was released from each of O2 and O9 LPS after strong acid hydrolysis (4N HCl, 100C, 45 min). On High-voltage paper electrophoresis (HVPE), X was separated into two components, X1 and X2, with mobilities of 1.54 and 1.80, respectively, relative to that of 2-keto-3-deoxy-octonate (KDO). X1 was identified as 2-keto-3-deoxy-5-phosphoryl-KDO (5-O-phosphoryl-KDO) on HVPE using, as a control, authentic 5-O-phosphoryl-KDO isolated from *Bordetella pertussis* (phase I). After treatment with alkaline-phosphatase, X1 as well as the authentic 5-O-phosphoryl-KDO disappeared and KDO was detected.

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Vibrio parahaemolyticus is a bacterium causing food poisoning through food agents such as marine products and more especially fresh fish and shellfish. It was discovered by Fujino et al[3] in 1950. Since that time, the same bacterium has kept being the cause of most food poisoning cases in Japan.

Vibrio parahaemolyticus (abbreviated hereafter as Vp) has on the basis of agglutination reactions and agglutinin absorption tests been serologically divided into 11 or 12 O-serum types[7,8]. Hisatsune et al[4] separated from the Vp O1 to O12 and refined the respective O-antigenic lipopolysaccharides (LPS) which are somatic antigens and for the first time elucidated quantitatively the sugar compositions. At the same time, they were first to find with regard to the Vp LPS other than O6LPS that the 2-keto-3-deoxyoctonate (KDO) contained as ordinary component in common gram-negative bacteria LPS are not detected in the Weissbach periodate-thiobarbituric acid reaction[12] (hydrolysis conditions: 0.02N sulfuric acid, 100 C, 20 min) used as ordinary method for the KDO reaction.

By the present study, we were the first to research the chemical properties of the LPS of Vp O2 and O9, and more particularly their molecular structure. We also separated the lipids A which are the centers of the endotoxin activity of both LPS, and elucidated the composition and presence system of their structural fatty acids. Moreover, we separated from the strong acid-hydrolysates (4N-HCl, 100 C, 45 min) of both LPS the periodate-thiobarbituric acid reaction-positive substance X, which differs clearly from KDO in the high-voltage filter paper electrophoresis and which had not yet been reported in the LPS of Vp, and we identified it for the first time as

5-O-phosphoryl KDO. On the basis of the obtained information, we discuss the structural background of the LPS molecules with regard to the LPS of Vp other than O6, in which KDO is undetected by the ordinary method.

We received the strains from Dr. Tatsuya OTA (Tokyo Municipal Health Research Center). We cultivated the bacteria during one night in a 3% table-salt-added bouillon culture (pH: 7.4), heated for 20 min at 120°C after which we washed the bacteria 3 times with distilled water. The LPS were extracted from the acetone-dried bacteria by the phenol-water method of Westphal[11]. We purified through repeated centrifugation (a total of 8 times) and RNase treatment. After hydrolyzing the O2LPS at 100°C with 5% acetic acid for 2 hours, and the O9LPS for 4.5 hours, we prepared the degraded polysaccharide (DPS) from the centrifugal supernatant. We prepared the lipids A by washing the centrifugal deposit 3 times with distilled water.

As for the neutral sugars, we hydrolyzed LPS with 2N-trifluoroacetic acid at 120 C for 1 h, after which, using a 3% ECNSS-M column as alditol-acetate derivative we performed the qualitative and quantitative analyses by gas chromatography. Regarding the amino sugars, we hydrolyzed the LPS with 4N hydrochloric acid at 100°C for 8 hours, after which, using a TABSORB column as total acetyl derivative, we performed the qualitative and quantitative analyses by gas chromatography. We performed the colorimetric quantitative determination of KDO by the periodate-thiobarbituric acid reaction of Weissbach[12]. We performed the qualitative and quantitative analyses of the fatty acids by gas chromatography as methyl esters derivatives. We performed the other quantitative reactions according to the norms

given in our former report[4]. For the high-voltage filter paper electrophoresis we used a pyridine/acetic acid/water (10:4:86, v/v/v, pH 5.3) solvent system on Whatman No. 1 filter paper and operated for 50 min at 50 v/cm. To detect the KDO on the filter paper, we used thiobarbituric acid reagents[10]. p. 542

The respective overall chemical compositions of O2 and O9LPS were as follows: total sugar (14.2% and 9.5%), reducing sugar (22.5% and 28.5%), total amino sugar (8.5% and 14.6%), total phosphorus (3.5% and 3.2%), proteins (3.5% and 2.8%) and total fat (26.0% and 25%).

Table 1 shows the sugar compositions of both LPS. Their common sugar components were glucose, heptose, glucosamine and uronic acid. Furthermore, galactose was found in O2LPS, and galactosamine and the rare sugars 4-amino-4, 6-dideoxyglucose were found in O9LPS. As a result of the gas chromatography analysis we discovered as heptose in O2LPS L-glycero-D-mannoheptose which is an ordinary component of common gram-negative bacteria LPS and the rare sugar D-glycero-D-mannoheptose.

Generally, the degraded polysaccharides (DPS) obtained by hydrolyzing with acetic acid (1%, 100 C, 1.5~2.5 hrs) the LPS of gram-negative bacteria such as salmonella and the colon bacillus are divided in 3 fractions by gel filtration with a Sephadex G-50 column. First the so-to-speak LPS total polysaccharide side chain part (Fr. I) formed by the bonding of the O-antigenic specific side chain and of the core polysaccharides in a void column is eluted. Thereafter, the core polysaccharides in imbibed volume (Fr. III) and the monosaccharides made from KDO and inorganic matters just ahead of the bed volume (Fr. IV) are eluted[5]. In the case of the DPS of the O1 cholera bacterium LPS, the fraction corresponding to the aforementioned Fr. I

is eluted somewhat later than the void volume (Fr. II)[5]. The DPS of the Vp O2 and O9LPS used in the present tests differ from those of salmonella or of the colon bacillus or from those of the cholera bacillum O1. Usually, Fr. I and Fr. II which are macromolecular fractions formed from the repeated polymers of the oligosaccharides by the bonding of cores to the O-antigenic specific polysaccharide side chains are not obtained. We obtained only the two Fr. III corresponding to the core polysaccharides and Fr. IV corresponding to the monosaccharides-inorganic matters fraction. This elution pattern was exactly the same as that of the R variant of the O1 cholera bacterium, that is to say of the DPS separated from the LPS of bacteria missing the O-antigenic specific side chain. The 2 O2 and O9 strains used to prepare the LPS clearly agglutinate specifically because of the respective antisera. Therefore, the above results seem to suggest that the O-antigenic specific chains of both LPS are generally remarkably shorter than those of the ordinary gram-negative bacteria or that they become sectioned on account of the mild acetic acid hydrolysis. The sugar compositions of Fr. III (Table 1) suggest that the core parts of the LPS of both bacteria are composed of heptose and of glucose and that galactose, glucosamine and uronic acid in O2LPS, and galactosamine, 4-amino-4, 6-dideoxyglucose and uronic acid in O9LPS are involved in the O-antigenic specific side chains of both bacteria.

The yield rate from the LPS of the O2 lipids A was 34.6% and p. 543
that from the O9 lipids A was 40.0%. The total lipid content of the former was 52.0% and that of the latter 55.4%. Table 2 shows the fatty acid compositions. We noted the following common component fatty acids

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in both lipids A viz. tetradecanoic acid ($C_{14:0}$), hexadecanoic acid ($C_{16:0}$), hexadecanoic acid ($C_{16:1}$), octadecanoic acid ($C_{18:1}$) and large quantities of 3-hydroxytetradecanoic acid (3-OH-$C_{14:0}$). We also discovered a small amount of octadecanoic acid ($C_{18:0}$) in the O2 lipids A and heptadecanoic acid ($C_{17:0}$) in the O9 lipids A. When examining by methanolysis using $NaOCH_3$ and by methylation using diazomethane, it seemed that in both lipids A there was a presence of 3-OH-$C_{14:0}$ bonded to N-amide and a presence of $C_{14:0}$, $C_{16:0}$ and $C_{16:1}$ bonded to O-esters. In the light of ^{13}C-NMR, ^{32}P-NMR-based analysis and of the chemical compositions (Table 1), we estimated that the O2 and O9 lipids A are always phosphatized at the positions 1 and 4 and that maybe similarly to the lipids A of salmonella and colon bacillus, they have a glucosamine-disaccharide backbone combined through β (1→6)-glucoside bonding.

KDO is an ordinary component in the LPS of common gram-negative bacteria. Except for O6 only, this KDO is not detected in the LPS of Vp by the ordinary method[12] (Hisatsune et al[4]). In the present study, we were the first to discover a substance (X) which is positive to the KDO reaction of Weissbach in the hydrolysates when the LPS of O2 and of O9 are hydrolyzed (100 C, 45 min) in strong acid (4N HCl) but which shows a behaviour clearly different from that of KDO in high-voltage filter paper electrophoresis.

Exactly like KDO, this X exhibits in the Weissbach reaction a coloring having a maximum absorption wavelength at 549 nm. However, in high-voltage filter paper electrophoresis, X was separated into the 2 components X_1 (relative shift degree with regard to KDO: $R_{KDO} = 1.54$) and X_2 ($R_{KDO} = 1.80$). Both are positive to the periodate-thiobarbituric

acid reaction[6,9] on filter paper. However, the degree of shift differed clearly from that of KDO (Fig. 1). Moreover, the degree of shift of this X_1 agreed perfectly with the shift degree (R_{KDO}) of the 5-O-phosphoryl KDO[2] separated from similar strong-acid hydrolysates of the whooping cough bacterium (phase I bacterium) LPS and of X[6] from the O3LPS of the cholera bacterium. Furthermore, these Vp O2, O9 separated by high-voltage vilter paper electrophoresis, as well as the X_1 from O3LPS of the cholera bacterium and the 5-O-phosphoryl KDO from the whooping cough bacterium LPS were treated separately with alkaline phosphatase (from Sigma; ammonium carbonate buffer solution, pH 9.6, 37 C, 16 hrs) after which high-voltage filter paper electrophoresis was performed. The spots corresponding respectively to X_1 and to 5-O-phosphoryl KDO disappeared, and we noted instead the appearance of new KDO spots (Fig. 1). On the basis of the above findings and of the results of methylation analysis vs. X_1, this X_1 in the strong hydrolysates of these O2 and O9LPS was for the first time identified in the present study as 5-O-phosphoryl KDO. The same identical substance p. 544 was also detected in similar strong acid hydrolysates of Vp O4LPS, and we separated the free KDO with alkaline phosphatase (Fig. 1).

In the inner core of the LPS of the bacteria of the intraintestinal bacteria family such as salmonella and the colon bacterium, the position 5 of KDO of 1 molecule is combined together with the position 1 of heptose and with glucoside. Meanwhile, the position 2 of the same KDO is bonded together with the position 6 of glucosamine-disaccharide-backbone of lipid A, and combines the heptose and lipid A. Moreover, the disaccharide of KDO (α-2-4→interlinked 3-deoxy-D-manno-octulosonic acid disaccharide) from this KDO molecule is indeed known to

be branched by a ketoside bond[1)]. In the Weissbach reaction, first these ketoside bonds are cut off by 0.02 M H_2SO_4 which is a weak acid. Thereafter, the β-formyl-pyruvate formed because of the periodate acidification of the free KDO of the 2 separated molecules develops color in the thiobarbituric acid. It is known that at this time, even if the glucoside bond is not cut, the KDO also whose position 5 is substituted by heptose shows in a monosubstitute situation a color development of a level which is the one of this reaction[9)]. In the case, however, of the LPS of Vp 02 and 09, instead of KDO, 5-O-phosphoryl KDO comes between heptose and lipid A. Its position 4 combines with the former in the glucoside bond, and the position 2 combines with the latter in the ketoside bond. It is also estimated that the KDO saccharide branched off in the ketoside bond from the above KDO and present in the LPS of intraintestinal bacteria family is missing. In such a structure, there is no liberated KDO in a free condition first because of the 0.02 M sulfuric acid heating. Because of this, the β-formyl-pyruvate to become chromogen because of periodate acidification does of course not form. The glucoside bond with the position 4 heptose of KDO is not severed under these heating conditions so that KDO is combined with heptose. Moreover, the position 5 phosphoric acid ester bond of 5-O-phosphoryl KDO is of course in opposition and resist vigorously the strong acid[2)]. Therefore both the position 4 and position 5 OH become present in the core part as substituted di-substitutes. However, the 4,5-di-substitutes of KDO differ from the 4-mono substitutes, and it has already been made clear that they show no color development in the periodate-thiobarbituric reaction[9)]. It seems

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that the above is the structural background of the Vp LPS in which KDO is undetected by the Weissbach reaction which is the ordinary method.

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Table 1 Sugar composition of LPS and their Lipid A isolated from *V. parahaemolyticus* O2 and O9, and the fraction obtained by Sephadex G-50 gel filtration of their "degrated polysaccharide" (DPS)

	Yield (%)	Glc	Gal	Hep	GlcN	GalN	4-amino-4, 6-dideoxy-Glc	Uronic acid	KDO
O2 LPS	–	9.1	2.6	8.9	8.3	–	–	6.0	nd[a]
Frc. III	23.4	13.1	8.3	20.7	–	–	–	17.3	nd
Frc. IV	11.7	–	–	–	–	–	–	4.0	nd
Lipid A	34.6	1.5	0.5	–	12.1	–	–	–	nd
O9 LPS	–	13.0	–	7.7	5.2	3.4	+	7.9	nd
Frc. III	25.4	9.1	–	20.3	–	11.1	+	21.7	nd
Frc. IV	12.0	tr[b]	–	–	–	–	–	8.8	nd
Lipid A	40.0	tr	–	–	14.0	–	–	–	nd

a) nd : not detected by the conventional Weissbach's periodate-thiobarbituric acid test under conventional hydrolytic condition[12]. b) tr : trace amount, less than 0.1%. Glc : Glucose, Gal : Galactose, Hep : Heptose, GlcN : Glucosamine, GalN : Galactosamine, KDO : 2-keto-3-deoxyoctonic acid.

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Table 2 Fatty acid composition of Lipid A isolated from *Vibrio parahaemolyticus* LPS (% of total fatty acid)

Fatty acid	Lipid A	
	O2	O9
C14:0	26.9	11.4
C15:0	tr[a)]	tr
C16:0	14.6	25.9
C16:1	14.6	20.7
C17:0	tr	2.2
C18:0	3.8	tr
C18:1	9.9	6.3
3OH—C14:0	30.2	33.6

a) tr : trace amont, less than 0.1%.

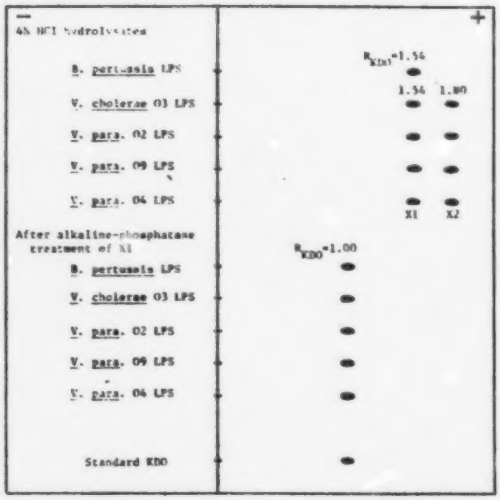

Fig. 1 High-voltage paper electrophoresis of X present in *Vibrio parahaemolyticus* LPS before and after alkaline-phosphatase treatment. *V. para* ; *Vibrio parahaemolyticus*.
High-voltage paper electrophoresis was performed with pyridine/acetic acid/water, 10:4:86, at 50 v/cm 50 min.

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